

Taxonomy/systematics

Atopostipes suicloacale gen. nov., sp. nov., isolated from an underground swine manure storage pitMichael A. Cotta^a, Terence R. Whitehead^{a,*}, Matthew D. Collins^b, Paul A. Lawson^b^a Fermentation Biotechnology Research Unit, National Center for Agricultural Utilization Research, USDA,¹ Agricultural Research Service, 1815 N. University Street, Peoria, IL 61604, USA^b School of Food Biosciences, University of Reading, Reading RG6 6AP, UK

Received 14 January 2004; received in revised form 26 March 2004; accepted 1 April 2004

Abstract

Phenotypic and molecular genetic studies were performed on an unknown facultative anaerobic, catalase-negative, non-spore-forming, rod-shaped bacterium isolated from a pig manure storage pit. The unknown bacterium was nutritionally fastidious with growth enhanced by the addition of rumen fluid and was phenotypically initially identified as an *Eubacterium* species. Comparative 16S rRNA gene sequencing studies, however, revealed that the unknown bacterium was phylogenetically distant from *Eubacterium limosum* (the type species of the genus *Eubacterium*) and related organisms. Phylogenetically, the unknown species displayed a close association with an uncultured organism from human subgingival plaque and formed an unknown sub-line within a cluster of organisms which includes *Alloiococcus otitis*, *Alkalibacterium olivoapovliticus*, *Allofustis seminis*, *Dolosigranulum pigrum*, and related organisms, within the low mol% G + C Gram-positive bacteria. Sequence divergence values of >8% with all known taxonomically recognised taxa, however, clearly indicates the novel bacterium represents a hitherto unknown genus. Based on both phenotypic and phylogenetic considerations, it is proposed that the unknown bacterium from pig manure be classified in a new genus and species, as *Atopostipes suicloacale* gen. nov., sp. nov. The type strain of *Atopostipes suicloacale* is PPC79^T=NRRL 23919^T=DSM 15692^T.

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Keywords: *Atopostipes suicloacale* sp. nov.; Taxonomy; 16S rRNA; Swine; Manure**1. Introduction**

Intensive swine production operations result in the generation of large quantities of manure which is increasingly being concentrated into smaller localities. Lagoon treatment or deep pit storage are among the most favoured methods used for the management of liquid swine manure. Odour emanating from the storage of swine manure can be a cause of considerable nuisance to the public. The odorous chemicals produced (e.g., ammonia, organic acids and alcohols, and sulphides) are largely due to incomplete digestion processes associated with anaerobic systems, but presently little is known

about the types of micro-organisms which reside in stored manure and are responsible for their production. In the course of a series of ongoing investigations [1,2] into the diversity of microbes which inhabit lagoons and manure pits, we have characterised a hitherto unknown, facultative anaerobic, asporogenous, rod-shaped organism within the *Clostridium* sub-phylum of the Gram-positive bacteria. In this article, we report the characteristics of this organism and the results of a polyphasic taxonomic study. Based on the presented findings, we describe a new genus and species, *Atopostipes suicloacale* gen. nov., sp. nov.

2. Materials and methods*2.1. Isolation and cultivation*

Isolate PPC79^T was recovered from a manure storage pit from a swine facility near Peoria, IL, USA where the

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¹ Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

feeder pigs were fed on a corn–soybean based diet. Samples were collected using a tank sampler (NASCO, Ft. Atkinson, WI) and transferred to Whirl-Pak sampling bags (NASDCO), and kept on ice until returned to the laboratory. Samples were transferred to an anaerobic glovebox, serially diluted and plated out onto agar medium containing 40% clarified rumen fluid as described [2]. Single colonies were repeatedly picked and streaked out until pure. Incubations were initially performed at 24°C under strictly anaerobic conditions to simulate the underground pit conditions [2]. Subsequent studies were carried out at varying temperatures. For morphological studies, the isolate was grown on routine growth medium (RGM, 2)-glucose agar plates with rumen fluid. For end products of metabolism, the isolate was grown on RGM-rumen fluid-glucose broth and fermentation products analysed using gas chromatographic and HPLC methodologies [3]. For utilisation studies, RGM-rumen fluid medium containing 0.4% of carbon test source was used. For biochemical tests performed using the API rapid ID32A system (BioMerieux, Durham, North Carolina), the organism was grown on RGM-rumen fluid agar with 0.2% glucose or RGM-rumen fluid agar with 0.2% lactose. Presence of spores was determined by visual examinations as well as incubations of the cultures in 95% ethanol followed by plating onto anaerobic agar medium.

2.2. DNA mol% G+C content determination

For the determination mol% of G+C content, DNA was isolated by the method of Saito and Miura [4]. The mol% G+C content was determined by thermal denaturation of DNA using a Beckman model DU 640 spectrophotometer equipped with a high performance temperature controller and T_m analysis software [5].

2.3. 16S rRNA gene sequencing and phylogenetic analysis

The 16S rRNA gene of the isolate was amplified by PCR using universal primers pA (positions 8 to 28, *Escherichia coli* numbering) and pH* (positions 1542–1522). The amplified product was purified by using a QIAquick PCR purification kit (QIAGEN Ltd., Dorking, UK) and directly sequenced using primers directed towards conserved positions of the rRNA gene and dRhodamine terminator cycle sequencing kit (PE Applied Biosystems, Inc., Foster City, CA, USA) and an automatic DNA sequencer (model 377; PE Applied Biosystems). DNA sequences were compared using database searches and the program FASTA [6], and the closest known relatives were determined. Closely related sequences were retrieved from EMBL and

aligned with the newly determined sequence using the program DNATools [7]. The resulting multiple sequence alignment had approximately 100 bases at the 5' end of the rRNA omitted from further analysis, because of alignment uncertainties due to the highly variable region V1, using the program GeneDoc [8]. A phylogenetic tree was re-constructed according to the neighbour-joining method [9] with the programs DNATools and TREEVIEW [10], and the stability of the groupings was estimated by bootstrap analysis (1000 replications).

2.4. Fatty acid and peptidoglycan analyses

Long-chain cellular fatty acids were analysed by the MIDI system (MIDI Inc., Newark, DE) according to manufacturer's instructions. Cell-wall murein was prepared by mechanical disruption of cells and acid hydrolysates analysed as described by Schleifer and Kandler [11] except that ascending thin-layer chromatography using cellulose sheets were used.

2.5. Nucleotide sequence accession number

The 16S rRNA gene sequence of strain PCC79^T has been deposited in GenBank under accession number AF445248.

3. Results and discussion

The unidentified organism recovered from a 10^{-7} dilution of pig manure slurry consisted of Gram-positive, short-rods. No visible spores were observed, and no growth was noted after incubation of cells in ethanol. The organism was facultatively anaerobic requiring rumen fluid for growth and was catalase-negative. The optimum growth temperature of the organism was 28–30°C, with 32°C its maximum temperature. After 72 h incubation anaerobically at 30°C, colonies were grey, convex, smooth, shiny and translucent. The unknown bacterium utilised amygdalin, cellobiose, esculin, glucose, lactose, maltose, mannose, raffinose, and sucrose; it also grew weakly on lactate. It did not utilise arabinose, cellulose, inulin, inositol, melibiose, rhamnose, sorbitol, trehalose, or xylose as carbon source. Using the commercially available API rapid ID32A system, the isolate grown on RGM-rumen fluid plus glucose plates, produced acid from mannose but not raffinose, and gave positive reactions for α -arabinosidase, α -galactosidase, β -glucosidase, and *N*-acetyl- β -glucosaminidase. The isolate was negative for urease, arginine dehydrolase, β -galactosidase, β -galactosidase-6-phosphate, α -glucosidase, α -arabinosidase, β -glucuronidase, glutamic acid decarboxylase, α -fucosidase, alkaline phosphatase, arginine arylamidase, proline arylamidase, leucyl-glycine

arylamidase, phenylalanine arylamidase, leucine arylamidase, pyroglutamic acid arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase, glutamyl-glutamic acid arylamidase, and serine arylamidase. The organism failed to reduce nitrate and did not produce indole. Using the same test system but with the isolate cultivated on RGM-rumen fluid plus lactose plates, the same results were obtained except β -galactosidase and β -galactosidase-6-phosphate were also detected. The DNA base composition of the strain was found to be 43.9 mol% G+C. The long-chain cellular fatty acids of the organism were found to be primarily of the unbranched saturated and unsaturated types, with *iso*- and *anteiso*-methyl branched forms present in very small amounts. The quantitative fatty acid data corresponded to: C_{10:0} (2.8%), C_{12:0} (2.1%), C_{14:0} (6.2%), *iso*-C_{15:0} (1.0%), *anteiso*-C_{15:0} (1.4%), C_{15:0} (1.6%), C_{16:1w9c} (37.6%), C_{16:0} (22.0%), *iso*-C_{17:1} (1.5%), *anteiso*-C_{17:1} (0.6%), C_{17:0} (1.1%), C_{18:1w9c} (9.3%), C_{18:1w7c} (0.6%) and C_{18:0} (12.2%). Analysis of the cell wall murein of PPC79^T showed the presence of an A4 α murein based on L-Lysine type: L-Lys-D-Asp.

To investigate the phylogenetic affinities of the unknown isolate, its almost complete 16S rRNA gene sequence was determined. Sequence database searches revealed the strain was a member of the low G+C *Clostridium* sub-phylum of the Gram-positive bacteria. Highest sequence similarity was shown with an uncultured rDNA clone originating from the human oral cavity, with the nearest named relatives to the unknown isolate corresponding to *Alkalibacterium olivoapovliticus*, *Alloiococcus otitis*, *Allofustis seminis*, *Carnobacterium* species, *Dolosigranulum pigrum*, *Enterococcus* species, *Granulicatella* species, and related catalase-negative organisms. Treeing analysis confirmed these affinities, with the unidentified bacterium forming a hitherto unknown sub-line amongst the aforementioned catalase-negative taxa (Fig. 1).

The unidentified, asporogenous, rod-shaped organism from pig manure phylogenetically did not correspond to any recognised Gram-positive bacterium. From the comparative 16S rRNA gene sequence analysis, it is evident that the unknown rod-shaped organism represents a previously unknown taxon. Phylogenetically, the novel bacterium, is a member of the *Clostridium* subphylum of the Gram-positive bacteria and forms an association with a cluster of organisms, which includes *Alkalibacterium olivoapovliticus*, *Alloiococcus otitis*, *Allofustis seminis*, *Dolosigranulum pigrum*, and some uncultured bacteria. The unidentified isolate formed a distinct sub-line amongst these organisms and displayed a relatively close affinity with an uncultured organism from human subgingival plaque [12]. Bootstrap analysis re-sampling reinforced the significance of this association (value 100% in 1000 tree

replications) although a sequence divergence value of 2.5% between the unidentified isolate and the uncultured organism was consistent with closely related, albeit genetically distinct, species. Amongst named taxa, the unidentified isolate displayed highest sequence relatedness to *Alkalibacterium olivoapovliticus* (91.9%), *Alloiococcus otitis* (90.5%), *Allofustis seminis* (90%), and *Dolosigranulum pigrum* (89.7%). The relatively high divergence values (>8%) together with the results of treeing analysis however showed the unidentified bacterium was only distantly related to these taxa and merits classification at a similar taxonomic rank (i.e., genus). It is pertinent to note that the separateness of the novel bacterium is strongly supported by phenotypic considerations. The isolate does not match any known Gram-positive organism phenotypically. The unidentified manure isolate differs markedly from *Alkalibacterium olivoapovliticus*, an obligate alkalophile (which grows within a pH range of ca. 8.5–10.8, and an optimum of pH ca. 9–10) originally recovered from wash waters of edible olives [13]. Similarly, the unidentified manure isolate differs from *Alloiococcus otitis*, *Allofustis seminis*, and *Dolosigranulum pigrum* in having a growth temperature optimum of 28–30°C and a maximum of 32°C. By contrast *Alloiococcus otitis*, *Allofustis seminis* and *Dolosigranulum pigrum* all grow optimally at 37°C [14–16]. In addition, unlike the unidentified isolate, cells of *Alloiococcus otitis* and *Dolosigranulum pigrum* are ovoid in shape [14,15]. *Alloiococcus otitis* further differs from the unidentified manure isolate in being aerobic [15]. Therefore, based on sequence divergence values of >8% with its nearest named phylogenetic relatives and the distinct sub-line formed by the novel bacterium, in concert with its quite distinct phenotypic characteristics, we are of the opinion that the unknown bacterium from swine manure merits assignment to a new genus, for which the name *Atopostipes suicloacale* is proposed. Although only a single strain of *Atopostipes suicloacale* is currently known, we consider the formal description of this species together with phenotypic criteria to aid its identification; will in the future facilitate its recognition in the laboratory, thereby permitting the recovery of additional strains of this species. In addition, we consider this formal description will encourage the search for additional species of the new genus, which undoubtedly exist in habitats other than swine manure.

3.1. Description of *Atopostipes* gen. nov.

Atopostipes [a.to.po.sti'pes. Gr. Adj. *atopos* having no place, strange, L. masc. n. *stipes* rod, N. L. masc. n. *Atopostipes* strange rod] cells consist of short rods. Cells stain Gram-positive and are non-motile, no spores are observed, facultatively anaerobic, catalase-negative. Acid is produced from glucose and some other

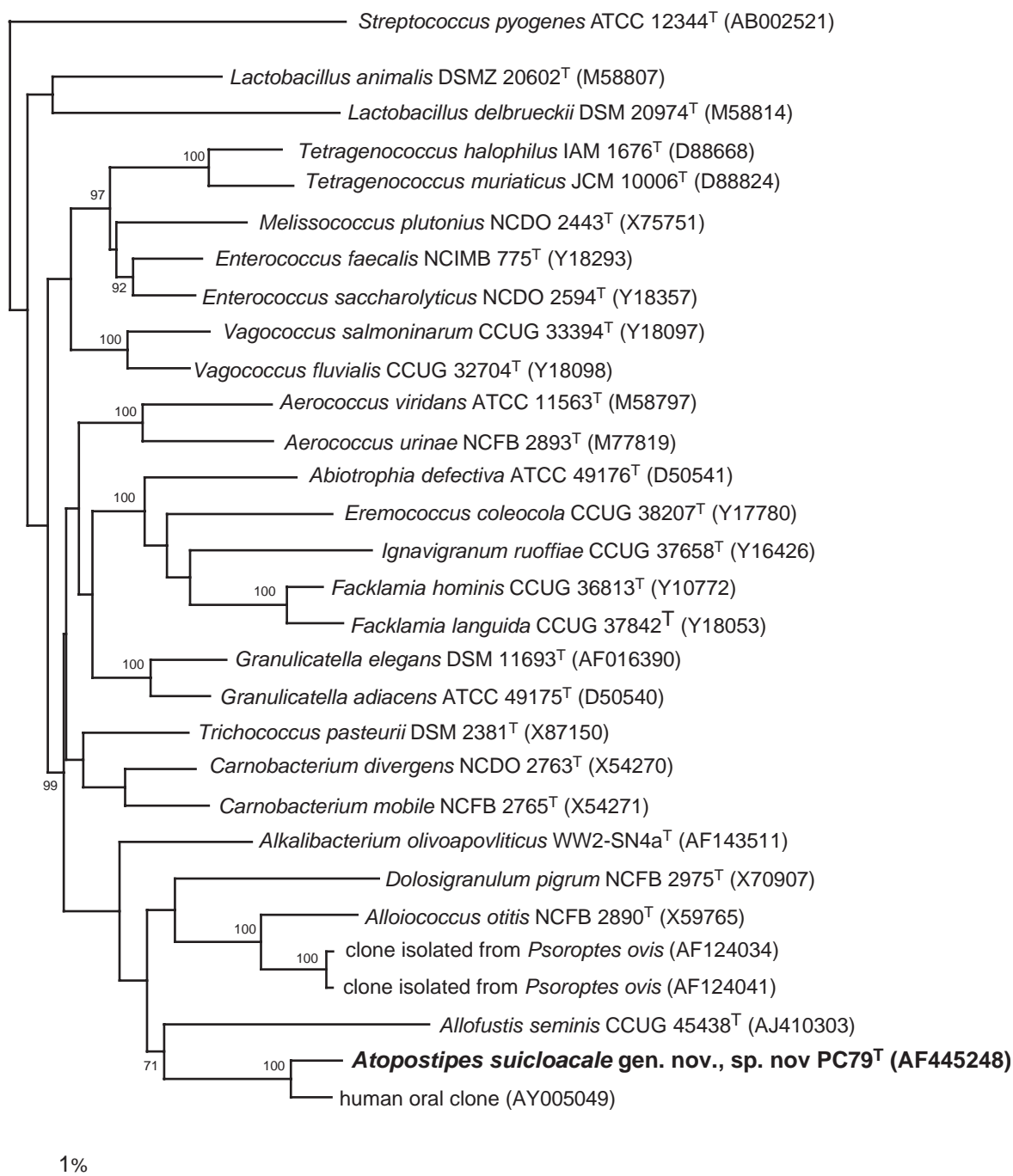


Fig. 1. Unrooted tree based on 16S rRNA showing the phylogenetic relationships of *Atopostipes suicloacale* gen. nov., sp. nov. The tree constructed using the neighbour-joining method is based on a comparison of approximately 1330 nucleotide positions. Bootstrap values, expressed as a percentage of 1000 replications, are shown at branching points. Bar = 1% sequence divergence.

carbohydrates. The end products of glucose metabolism are lactate, acetate, and formate, urease-negative. Nitrate is not reduced. Indole is not formed. The long-chain cellular fatty acids of the organism were found to be predominately of the straight-chain saturated and monounsaturated types. Cell wall murein is based on L-Lys variation A4 α (type L-Lys-D-Asp). The type species is *Atopostipes suicloacale*. The G + C content of genomic DNA of the type species is 43.9 mol%.

3.2. Description of *Atopostipes suicloacale* sp. nov.

Atopostipes suicloacale [su.i.clo.a.ca'le. L. n. *sus* pig, L. n. adj. *cloacale* pertaining to a sewer (manure canal), N. L. masc. n. *suicloacale* from pig manure] cells are short-rods which stain Gram-positive. No spores are observed, and cells are non-motile. Colonies are pin-head to 0.5 mm in diameter, grey, smooth, circular, and entire after 48 h incubation on Brain heart infusion agar

supplemented with 10% rumen fluid. Optimum growth temperature is 28–30°C; no growth above 32°C; facultatively anaerobic, and catalase-negative. Produces acid from glucose; end products of glucose metabolism are lactate, acetate, and formate. Amygdalin, cellobiose, esculin, glucose, lactose, maltose, mannose, raffinose, and sucrose are utilised as energy source; grows weakly on lactate. Arabinose, cellulose, inulin, inositol, melibiose, rhamnose, sorbitol, trehalose, and xylose are not utilized as energy source. Using the API rapid ID 32A kit, α -arabinosidase, α -galactosidase, β -glucosidase, and *N*-acetyl- β -glucosaminidase are detected from cells grown on RGM-rumen fluid plus glucose; in addition, β -galactosidase and β -galactosidase-6-phosphate are detected from cells grown on RGM-rumen fluid with lactose. Arginine dihydrolase, arginine arylamidase, alkaline phosphatase, alanine arylamidase, α -fucosidase, α -glucosidase, β -glucuronidase, glycine arylamidase, glutamic acid decarboxylase, glutamyl glutamic acid arylamidase, histidine arylamidase, leucine arylamidase, leucyl glycine arylamidase, phenyl alanine arylamidase, proline arylamidase, pyroglutamic acid arylamidase, serine arylamidase, tyrosine arylamidase, and urease are not detected. Nitrate is not reduced. Indole is not formed. The major long-chain cellular fatty acids are C_{14:0}, C_{16:0}, C_{16:1}, C_{18:0} and C_{18:1}. The DNA base composition is 43.9 mol% G+C. Other chemotaxonomic properties are as described for the genus. The type strain is PPC79^T=NRRL 23919^T=DSMZ15692^T. Isolated from swine manure slurry.

References

- [1] Whitehead TR, Cotta MA. Characterisation and comparison of microbial populations in swine faeces and manure storage pits by 16S rDNA gene sequence analysis. *Anaerobe* 2000;7:181–7.
- [2] Cotta MA, Whitehead TR. Isolation, characterization, and comparison of bacteria from swine feces and manure storage pits. *Environ Microbiol* 2003;5:737–45.
- [3] Miller DN. Accumulation and composition of odorous compounds in feedlot soils under aerobic, fermentative, anaerobic respiratory conditions. *J Anim Sci* 2001;79:2503–12.
- [4] Saito H, Miura H. Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochem Biophys Acta* 1963;22:619–29.
- [5] Johnson JL. Similarity analysis of DNAs. In: Gerhardt P, Murray RGE, Wood WA, Kreig NR, editors. *Methods for general and molecular bacteriology*. Washington, DC: ASM; 1994. p. 656–82.
- [6] Pearson WR, Lipman DJ. Rapid and sensitive protein similarity searches. *Science* 1985;227:1435–41.
- [7] Rasmussen SW. DNATools, a software package for DNA sequence analysis. Copenhagen: Carlsberg Laboratory; 1995.
- [8] Nicholas KB, Nicholas Jr HB, Deerfield II DW. GeneDoc: analysis and visualization of genetic variation. *EMBNW News* 1997;4:14.
- [9] Saitou N, Nei M. The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987;4: 406–25.
- [10] Page RDM. TREEVIEW: an application to display phylogenetic trees on personal computer. *Comp Appl Biosci* 1996;12:357–8.
- [11] Schleifer KH, Kandler O. Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* 1972;36:407–77.
- [12] Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, Sahasrabudhe A, Dewhirst FE. Bacterial diversity in human subgingival plaque. *J Bacteriol* 2001;183:3770–83.
- [13] Ntougias S, Russell NJ. *Alkalibacterium olivoapovlenticus* gen. nov., sp. nov., a new obligately alkaliphilic bacterium isolated from edible-olive wash waters. *Int J Syst Evol Microbiol* 2001;51: 1161–70.
- [14] Aguirre M, Morrison D, Cookson BD, Gay FW, Collins MD. Phenotypic and phylogenetic characterisation of some *Gemella*-like organisms from human infections: description of *Dolosigranulum pigrum* gen. nov., sp. nov. *J Appl Bacteriol* 1993;75:608–12.
- [15] Aguirre M, Collins MD. Phylogenetic analysis of *Alloiooccus otitis* gen. nov., sp. nov., an organism from human middle ear fluid. *Int J Syst Bacteriol* 1992;42:79–83.
- [16] Collins MD, Higgins R, Messier S, Fortin M, Hutson RA, Lawson PA, Falsen E. *Allofustis seminis* gen. nov., sp. nov., a novel Gram-positive, catalase-negative, rod-shaped bacterium from pig semen. *Int J Syst Evol Microbiol* 2003;53:811–4.